

**Preliminary Amendment**

Page 5

Applicants: Przybyla et al.

Serial No.: Unknown (Int'l. Application No. PCT/US99/31176)

Filed: On Even Date Herewith (Int'l. Filing Date: 12/29/99)

Title: RUBREDOXIN FUSION PROTEINS, PROTEIN EXPRESSION SYSTEM AND METHODS

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resolution between  $\beta$ -amyloid 1-42 and the various contaminating peaks. The  $\beta$ -amyloid 1-42 peptide isolated by this protocol was found to be pure as judged by mass spectrometry and was free of chemical modifications. However, this method poses a problem in that the temperatures used are very close to the boiling point of acetonitrile and further, heating a scale up preparatory column is a long and expensive proposition. Moreover, it is difficult to work at a pH above about pH 6 with silica based resins since at high temperatures silica tends to degrade at a pH above 5.

**In the Claims**

Please cancel claims 1-44; and add new claims 45-114. The new claims are provided below in clean form. Per 37 C.F.R. §1.121, the added claims are also shown in Appendix A (for convenience, all pending claims are provided in Appendix A).

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45. (New) A recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal polypeptide, when not fused to the rubredoxin constituent, is insoluble or forms inclusion bodies in a recombinant expression system.

46. (New) The recombinant polynucleotide of claim 45 wherein the N-terminal rubredoxin constituent is cleavably linked to the C-terminal fused polypeptide.

47. (New) The recombinant polynucleotide of claim 45 wherein the rubredoxin fusion protein further comprises an intervening spacer region positioned between the N-terminal rubredoxin constituent and the C-terminal fused polypeptide.

**Preliminary Amendment**

Page 6

Applicants: Przybyla et al.

Serial No.: Unknown (Int'l. Application No. PCT/US99/31176)

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48. (New) The recombinant polynucleotide of claim <sup>47</sup>45 wherein the intervening spacer region comprises at least one component selected from the group consisting of a proteolytic cleavage site and an affinity purification sequence.

49. (New) A recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the fusion protein binds a divalent cation and is chromogenic.

50. (New) An expression vector comprising:

a nucleotide sequence encoding rubredoxin or a biologically active analogue, fragment, or modification thereof;

an intervening nucleotide sequence encoding a spacer region; and

a multiple cloning region comprising at least one restriction endonuclease recognition site.

51. (New) The expression vector of claim 50 wherein the intervening nucleotide sequence comprises all or a portion of the multiple cloning region.

52. (New) The expression vector of claim 51 which is pRUBEX3, wherein pRUBEX3 comprises a nucleotide sequence encoding an affinity tag having at least one amino acid sequence selected from the group consisting of His-His-His-His-His-His (SEQ ID NO:4) and His-Gly-Leu-His (SEQ ID NO:7).

53. (New) The expression vector of claim 50 wherein the intervening nucleotide sequence encodes at least one of a proteolytic cleavage site and an affinity purification sequence.

54. (New) An expression vector comprising a promoter operably linked to a nucleotide

**Preliminary Amendment**

Page 7

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Title: RUBREDOXIN FUSION PROTEINS, PROTEIN EXPRESSION SYSTEM AND METHODS

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sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal polypeptide, when not fused to the rubredoxin constituent, is insoluble or forms inclusion bodies in a recombinant expression system.

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55. (New) An expression vector comprising a promoter operably linked to a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the fusion protein binds a divalent cation and is chromogenic.

56. (New) A host cell transformed with an expression vector comprising a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal polypeptide, when not fused to the rubredoxin constituent, is insoluble or forms inclusion bodies in a recombinant expression system.

57. (New) The host cell of claim 56 which is a bacterial cell.

58. (New) A host cell transformed with an expression vector comprising a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the fusion protein binds a divalent cation and is chromogenic.

59. (New) The host cell of claim 58 which is a bacterial cell.

60. (New) A method for making a rubredoxin fusion protein comprising:

**Preliminary Amendment**

**Page 8**

Applicants: Przybyla et al.

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---

(a) introducing into a host cell a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide; and

(b) expressing the fusion protein in the host cell, wherein the fusion protein binds a divalent cation and is chromogenic.

61. (New) The method of claim 60 wherein the host cell contains or is supplied with at least one isotopically labeled amino acid or precursor compound, wherein the fusion protein expressed in the host cell in step (b) is isotopically labeled.

62. (New) The method of claim 61 wherein the host cell is an amino acid auxotroph.

63. (New) The method of claim 61 wherein the fused polypeptide is isotopically labeled with at least one of  $^{35}\text{S}$ ,  $^{13}\text{C}$ , or  $^{15}\text{N}$ .

64. (New) The method of claim 60 wherein the C-terminal fused polypeptide comprises an amyloid peptide or a biologically active fragment, modification or analogue thereof.

65. (New) The method of claim 60 further comprising (c) removing the fusion protein from the host cell.

66. (New) The method of claim 65 further comprising (d) purifying the fusion protein.

67. (New) The method of claim 66 wherein step (d) comprises visually tracking the location of the fusion protein.

**Preliminary Amendment**

**Page 9**

Applicants: Przybyla et al.

Serial No.: Unknown (Int'l. Application No. PCT/US99/31176)

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---

68. (New) The method of claim 66 wherein step (d) comprising purifying the fusion protein using reverse phase chromatography at temperatures between about 45°C and about 65°C.

69. (New) The method of claim 66 further comprising (e) cleaving the fusion protein to yield the rubredoxin constituent and the polypeptide.

70. (New) The method of claim 69 further comprising (f) purifying the polypeptide using reverse phase chromatography at temperatures between about 45°C and about 65°C.

71. (New) A method for making a polypeptide which, when not fused to a rubredoxin constituent, is insoluble or forms inclusion bodies in a recombinant expression system, the method comprising:

(a) introducing into a host cell a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal polypeptide, when not fused to the rubredoxin constituent, is insoluble or forms inclusion bodies in a recombinant expression system; and

(b) expressing the fusion protein in the host cell.

72. (New) The method of claim 71 wherein the host cell contains or is supplied with at least one isotopically labeled amino acid or precursor compound, wherein the fused protein expressed in the host cell in step (b) is isotopically labeled.

73. (New) The method of claim 72 wherein the host cell is an amino acid auxotroph.

74. (New) The method of claim 72 wherein the fused protein is isotopically labeled with at least one of <sup>35</sup>S, <sup>13</sup>C, or <sup>15</sup>N.

75. (New) The method of claim 72 wherein the C-terminal fused polypeptide comprises an amyloid peptide or a biologically active fragment, modification or analogue thereof.

76. (New) The method of claim 71 further comprising (c) removing the fusion protein from the host cell.

77. (New) The method of claim 71 further comprising (d) purifying the fusion protein.

78. (New) The method of claim 71 wherein step (d) comprises visually tracking the location of the fusion protein.

79. (New) The method of claim 71 wherein the fusion protein is purified using reverse phase chromatography at temperatures between about 45°C and about 65°C.

80. (New) The method of claim 77 further comprising (e) cleaving the fusion protein to yield the rubredoxin constituent and the polypeptide.

81. (New) The method of claim 80 further comprising (f) purifying the polypeptide using reverse phase chromatography at temperatures between about 45°C and about 65°C.

82. (New) A method for making a rubredoxin- $\beta$ -amyloid fusion protein comprising:

(a) introducing into a host cell a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused  $\beta$ -amyloid peptide, wherein the host cell contains or is supplied with at least one isotopically labeled amino acid or precursor compound; and

**Preliminary Amendment**

**Page 11**

Applicants: Przybyla et al.

Serial No.: Unknown (Int'l. Application No. PCT/US99/31176)

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Title: RUBREDOXIN FUSION PROTEINS, PROTEIN EXPRESSION SYSTEM AND METHODS

---

(b) expressing a rubredoxin- $\beta$ -amyloid fusion protein in the host cell wherein the fused  $\beta$ -amyloid peptide is uniformly isotopically labeled.

83. (New) The method of claim 82 wherein the rubredoxin- $\beta$ -amyloid fusion protein is uniformly labeled with at least one of  $^{35}\text{S}$  and  $^{15}\text{N}$ .

84. (New) The method of claim 82 further comprising (c) removing the rubredoxin- $\beta$ -amyloid fusion protein from the host cell and (d) purifying the rubredoxin- $\beta$ -amyloid fusion protein using reverse phase chromatography at temperatures between about  $45^\circ\text{C}$  and about  $65^\circ\text{C}$ .

85. (New) The method of claim 82 further comprising cleaving the rubredoxin- $\beta$ -amyloid fusion protein to yield the rubredoxin constituent and the  $\beta$ -amyloid peptide.

86. (New) The method of claim 85 further comprising purifying the  $\beta$ -amyloid peptide using reverse phase chromatography at temperatures between about  $45^\circ\text{C}$  and about  $65^\circ\text{C}$ .

87. (New) A method for making a rubredoxin- $\beta$ -amyloid fusion protein comprising:

(a) introducing into a host cell a recombinant polynucleotide comprising a nucleotide sequence encoding a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused  $\beta$ -amyloid peptide, wherein the host cell contains or is supplied with at least one isotopically labeled amino acid or precursor compound selected from the group consisting of  $^{35}\text{S}$ -methionine and an  $^{15}\text{N}$ -labeled precursor compound; and

(b) expressing a rubredoxin- $\beta$ -amyloid fusion protein in the host cell wherein the fused  $\beta$ -amyloid peptide is uniformly labeled with at least one of  $^{35}\text{S}$  and  $^{15}\text{N}$ .

88. (New) A rubredoxin fusion protein comprising a chromogenic N-terminal rubredoxin constituent and a C-terminal fused polypeptide.

**Preliminary Amendment**

Page 12

Applicants: Przybyla et al.

Serial No.: Unknown (Int'l. Application No. PCT/US99/31176)

Filed: On Even Date Herewith (Int'l. Filing Date: 12/29/99)

Title: RUBREDOXIN FUSION PROTEINS, PROTEIN EXPRESSION SYSTEM AND METHODS

---

89. (New) A rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal polypeptide, when not fused to the rubredoxin constituent, is insoluble or forms inclusion bodies in a recombinant expression system.

90. (New) The rubredoxin fusion protein of claim 89 which is soluble when overexpressed in a host cell.

91. (New) A rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide selected from the group consisting of an amyloid peptide, leptin, proinsulin, trypsin inhibitor, the extracellular domain of luteinizing hormone receptor, and a biologically active fragment, modification or analogue of any of the preceding polypeptides.

92. (New) A rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal fused polypeptide comprises an amyloid peptide or a biologically active fragment, modification or analogue thereof.

93. (New) The rubredoxin fusion protein of claim 92 comprising a detectable label.

94. (New) The rubredoxin fusion protein of claim 93 wherein the detectable label is a mass isotope or a radioisotope.

95. (New) The rubredoxin fusion protein of claim 93 wherein the detectable label is selected from the group consisting of  $^{35}\text{S}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$ .



**Preliminary Amendment**

Page 13

Applicants: Przybyla et al.

Serial No.: Unknown (Int'l. Application No. PCT/US99/31176)

Filed: On Even Date Herewith (Int'l. Filing Date: 12/29/99)

Title: RUBREDOXIN FUSION PROTEINS, PROTEIN EXPRESSION SYSTEM AND METHODS

---

96. (New) The rubredoxin fusion protein of claim 95 which is uniformly labeled with the detectable label.

97. (New) The rubredoxin fusion protein of claim 95 which is uniformly labeled with both  $^{35}\text{S}$  and  $^{15}\text{N}$ .

98. (New) The rubredoxin fusion protein of claim 92 wherein the N-terminal rubredoxin constituent is cleavably linked to the C-terminal fused polypeptide.

99. (New) A rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide, wherein the C-terminal fused polypeptide comprises an  $^{35}\text{S}$ -labeled  $\beta$ -amyloid peptide.

100. (New) The rubredoxin fusion protein of claim 99 wherein the C-terminal fused polypeptide comprises an  $^{35}\text{S}$ -methionine-labeled  $\beta$ -amyloid peptide.

101. (New) The rubredoxin fusion protein of claim 99 wherein the C-terminal fused polypeptide comprises an  $^{35}\text{S}$ -labeled 1-42  $\beta$ -amyloid peptide.

102. (New) The rubredoxin fusion protein of claim 99 wherein  $\beta$ -amyloid peptide is uniformly labeled with  $^{35}\text{S}$ .

103. (New) The rubredoxin fusion protein of claim 99 wherein  $\beta$ -amyloid peptide is uniformly labeled with  $^{35}\text{S}$  and  $^{15}\text{N}$ .

104. (New) An  $^{35}\text{S}$ -labeled  $\beta$ -amyloid peptide.

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**Preliminary Amendment**

**Page 14**

Applicants: Przybyla et al.

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105. (New) An  $^{35}\text{S}$ -methionine-labeled  $\beta$ -amyloid peptide.

106. (New) The  $^{35}\text{S}$ -methionine labeled  $\beta$ -amyloid peptide selected from the group consisting of an  $^{35}\text{S}$ -methionine labeled 1-42  $\beta$ -amyloid peptide and an  $^{35}\text{S}$ -methionine labeled 1-40  $\beta$ -amyloid peptide.

107. (New) A  $\beta$ -amyloid peptide that is uniformly labeled with  $^{35}\text{S}$  and  $^{15}\text{N}$ .

108. (New) A method for making an antibody comprising eliciting in a host cell an immune response to an antigen comprising a rubredoxin fusion protein comprising a N-terminal rubredoxin constituent and a C-terminal fused polypeptide to yield antibodies to the fused polypeptide.

109. (New) The method of claim 108 wherein the antibody is a polyclonal antibody.

110. (New) The method of claim 108 wherein the antibody is a monoclonal antibody.

111. (New) The method of claim 108 where the antibody is not cross-reactive with rubredoxin.

112. (New) A vaccine comprising:

at least one component selected from the group consisting of:

- (a) a rubredoxin fusion protein comprising an N-terminal rubredoxin constituent and a C-terminal fused polypeptide; and
  - (b) a polynucleotide comprising a nucleotide sequence encoding said rubredoxin fusion protein; and
- a pharmaceutically acceptable carrier.

**Preliminary Amendment**

**Page 15**

Applicants: Przybyla et al.

Serial No.: Unknown (Int'l. Application No. PCT/US99/31176)

Filed: On Even Date Herewith (Int'l. Filing Date: 12/29/99)

Title: RUBREDOXIN FUSION PROTEINS, PROTEIN EXPRESSION SYSTEM AND METHODS

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113. (New) The vaccine of claim 112 wherein the N-terminal rubredoxin constituent is directly linked to the C-terminal fused polypeptide.

114. (New) The vaccine of claim 112 further comprising an adjuvant.

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